LSAMP AND *NORE1* DOWN-REGULATION IN CLEAR CELL RENAL CELL CARCINOMAS

BACKGROUND OF THE INVENTION

Field of the Invention

5   The present invention in the field of genetics and medicine relates to the under-expression of two genes, *LSAMP* and *NORE1* in clear cell renal cell carcinoma (CC-RCC) tissue and exploitation of this property in methods for detecting or treating this type of cancer.

Description of the Background Art

Renal carcinoma is known to have different histological types, with distinct genetic
10   profiles (Storkel *et al.*, 1997). Worldwide, approximately 150,000 people are diagnosed with renal carcinoma, resulting in 78,000 deaths annually (Zbar *et al.*, 2002). The most common type is clear cell renal cell carcinoma (CC-RCC). Studies of familial CC-RCC have led to the identification of important tumor suppressor genes such as *VHL* (Latif *et al.*, 1993). Recently, position cloning also resulted in the discovery of other kidney cancer-related genes *BHD*, *FH*,
15   and *HRPT2* (Nickerson *et al.*, 2002; Tomlinson *et al.*, 2002; Carpten *et al.*, 2002). While hereditary CC-RCCs are mainly attributed to *VHL* mutations, there are known CC-RCC families and a significant proportion of sporadic CC-RCCs that are not associated with the *VHL* (Teh *et al.*, 1997; Woodward *et al.*, 2000), thus pointing to the existence of other CC-RCC-related genes. Since some CC-RCC families are associated with balanced chromosomal translocations,
20   the translocation breakpoint-spanning genes are likely CC-RCC-related candidate genes. The first CC-RCC family with a balanced chromosomal translocation t(3;8)(p14;q24) was described by Cohen *et al.* (1979). To date, at least eight such hereditary CC-RCC-related chromosomal translocation families have been reported (Cohen *et al.*, 1979; Kovacs *et al.*, 1988; Kovacs *et al.*, 1989; Koolen *et al.*, 1998; van Kessel *et al.*, 2001; Podolski *et al.*, 2001; Kanayama *et al.*, 2001).
25   Interestingly, translocation in all these CC-RCC families is linked to chromosome 3, making constitutional chromosome 3 translocation a predisposing factor (vas Kessel *et al.*, 1999; Bodmer *et al.*, 1998; Bodmer *et al.*, 2002c). The subsequent observation of the loss of translocation derivative chromosome 3 (der(3) chromosome) and somatic *VHL* mutations in a proportion of familial tumors led to the proposal of a three-step model of CC-RCC
30   tumorigenesis (Schmidt *et al.*, 1995; Bodmer *et al.*, 1998; Bodmer *et al.*, 2002c): initial constitutional chromosome 3 translocation, subsequent somatic loss of the der(3) chromosome

leading to the loss of a copy of *VHL*, and a third hit in the form of random somatic mutation in the second *VHL* allele. However, loss of the der(3) chromosome was observed only in a subset of the examined samples. Most of the analyzed familial tumors with loss of the der(3) did not carry *VHL* mutations. Furthermore, neither der(3) loss nor *VHL* mutations were observed in

5 several tumor biopsies in the affected families (Eleveld *et al.*, 2001; Bodmer *et al.*, 2002b). These observations suggest that the breakpoint-spanning genes in the familial RCC-associated chromosome 3 translocations are also likely implicated in RCC tumorigenesis or act synergistically in the above model in the form of genetic and/or epigenetic alternations.

Analysis of the constitutional t(3;8)(p14;q24) translocation associated with familial CC-

10 RCC led to the identification and extensive investigation of the breakpoint-spanning gene *FHIT* (fragile histidine triad) on 3p14 (Ohta *et al.*, 1996). *FHIT* is thought to be a putative tumor suppressor gene, and aberrant *FHIT* transcripts and *FHIT* genomic lesions were observed in a variety of primary tumors and tumor-derived cell lines (Ohta *et al.*, 1996; Siprashvili *et al.*, 1997; Druck *et al.*, 1997). The partner breakpoint-spanning gene *TRC8* on the chromosome 8

15 shows high homology to the *Drosophila* patched (*PTCH*) gene and probably also functions as a tumor suppressor (Gemmill *et al.*, 2002). Also, another two breakpoint-spanning genes, *DIRC1* on chromosome 2q33 and *DIRC2* on 3q21, disrupted respectively in t(2;3)(q33;q21) and t(2;3)(q35;q21) breakpoints, have been identified (Druck *et al.*, 2001; Bodmer *et al.*, 2002a). The role of these genes in CC-RCC tumorigenesis remains to be determined.

20 The present inventors describe here the positional cloning of the t(1;3)(q32.1;q13.3) chromosomal breakpoints and the identification of two breakpoint-spanning genes, *LSAMP* on 3q13.3 and *NORE1* on 1q32.1, in a previously reported Japanese hereditary CC-RCC family (Kanayama *et al.*, 2001). *LSAMP* (limbic-system-associated membrane protein gene) encodes a neuronal surface glycoprotein that belongs to the IgLONs (immunoglobulin LSAMP,

25 OPCML/OBCAM, and neurotrimin) family and is distributed in cortical and subcortical regions of the limbic system (Pimenta *et al.*, 1996). To date, very little is known about *LSAMP* and its biological role remains unclear. However, its family partner gene *OPCML/OBCAM* on 11q25 was recently found to be epigenetically inactivated and was regarded as a candidate TSG in epithelial ovarian cancer (Sellar *et al.*, 2003). *NORE1* was recently identified as a homolog of

30 the tumor suppressor gene *RASSF1* at 3p21.3, which is frequently inactivated via promoter hypermethylation in a variety of human tumors (Dammann *et al.*, 2000; Tommasi *et al.*, 2002). The mouse counterpart Nore1 is a Ras effector (Vavvas *et al.*, 1998).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 and its subparts show mapping of the of the t(1;3) breakpoints on chromosomes 1q32.1 and 3q13.3 by FISH.

Fig. 1A-1 shows the construction of a contig of nine BAC clones in a 3.6-cM region of
5   1q32.1 (left panel, labeled "1q"). The RP11-54L22 was first found to span the breakpoint by FISH split assay. Overlapped BAC clones (CTD-2245C1, -2321B11, and -2278G17) also showed split signals and confine the der(1) breakpoint region to about 30 kb (dotted box).

Fig. 1A-2 shows the similar establishment of a contig of ten BAC clones within a 5-cM region of 3q13.3 (right panel, labeled "3q"). The breakpoint was found within RP11-281N16
10   and was further mapped to a about 30-kb region (dotted box) using overlapping clones (RP11-149B11, CTD-2246M24, and -2514L8).

Fig. 1B shows fine mapping of the 1q32.1 and 3q13.3 breakpoints by Southern blot analysis and restriction mapping. Nine specific DNA probes (4-10 kb) flanking the 1q breakpoint were synthesized by long-range PCR with specific primers from known-sequence
15   RP11-54L22 and -281N16. Southern blot analyses showed that a 5.6-kb 1q-p4 probe (**Fig. 1B-1** - left panel, labeled RP11-54L22 clone") and a 6.2-kb 3q-p2 probe (**Fig. 1B-2** - right panel, labeled "RP11-281N16 clone") span the respective 1q32.1 and 3q13.31 breakpoints, which narrowed both breakpoint regions to approximately 6 kb. Restriction mapping refined the 1q and the 3q breakpoints to about 1.5-kb (Fig. 1B-1, left panel) and 2-kb regions, respectively
20   (Fig. 1B-2, right panel).

Fig. 1C shows representative Southern-blot analyses from both chromosomes showing distinct aberrant bands (indicated by arrowheads) after restriction digestion. DNA from two normal controls (N1, N2) and two patients (FRCC3 and FRCC5) were completely digested and subjected to DNA hybridization analysis. **Fig. 1C-1** shows the Southern blot from chromosome
25   1q32.1; **Fig. 1C-2** shows the Southern blot from chromosome 3q13.3.

Figure 2 shows the cloning of both der(1) (1q32.1) and der(3) (3q13.31) breakpoints through long-range PCR and DNA sequencing.

Fig. 2A shows amplification of der(1) and der(3) breakpoints via long-range PCR. A 2.15-kb der(1) breakpoint fragment (der(1)-BP) and a 3.25-kb der(3) breakpoint fragment
30   (der(3)-BP) were amplified. The breakpoint fragments were sequenced and are shown in the lowest boxes. The normal sequences around the breakpoints on 1q32.1 and 3q13.31 are also

shown for comparison. The uppercase sequences are from 1q32.1 and the lowercase sequences are from 3q13.31. The sequences in red on 3q13.31 are deleted from the breakpoints.

Fig. 2B is a schematic illustration of the identification of breakpoint-spanning genes. The translocation breakpoints occur within intron 2 of both breakpoint-spanning genes *LSAMP* at
5   1q32.1 and *NORE1* at 3q13.31, which is accompanied by loss of 52 or 54 bp (red sequences in panel A) from *LSAMP* and of 2 or 0 bp from *NORE1*. An insertion of nucleotide G (ins G) in the breakpoint junction and a loss of 2 bp (delTG) in *LSAMP* in the distal part of breakpoint were also observed. *NORE1* has two isoforms, *NORE1A* and *NORE1B*. *LSAMP* contains seven exons and sits in the reverse strand of chromosome 3.

10   Figure 3 shows lower expression and promoter methylation of *LSAMP* and *NORE1A* in RCC cell lines and sporadic RCC tumors.

Fig. 3A shows that the expression of LSAMP (Fig. 3A-1) and NORE1A (Fig. 3A-3) in nine RCC cell lines and sporadic tumors (LSAMP; 0.06 ± 0.06 for cell lines and 0.05 ± 0.07 for tumors; NORE1A: 0.19 ± 0.10 for cell lines and 0.27 ± 0.12 for tumors) is significantly lower
15   than that in nine normal kidney tissues (LSAMP; 0.77 ± 0.28; NORE1A; 1.03 ± 0.53) using real-time PCR assay (*t*-test of SSPS, $p < 0.001$). Fig. 3A-2 and Fig. 3A-4 (Right panels) show eight RCC cell lines which were demethylated using 5-aza-CdR; the expression of both *LSAMP* (Fig. 3A-2) and *NORE1A* (Fig. 3A-4) was significantly increased in each line (LSAMP; untreated, 0.35 ± 0.23; 5-aza-CdR, 1.02 ± 0.33; NORE1; untreated, 0.03 ± 0.02; 5-aza-CdR, 0.24 ± 0.10)
20   (*t*-test of SSPS, $p < 0.001$).

Fig. 3B shows methylation analysis of the *LSAMP* promoter. Bisulfite-treated DNA from 53 matched pairs of human CC-RCC tumors and normal DNA samples, 9 RCC cell lines, 2 t(1;3)-positive lymphoblastoid cell lines, and 2 control lymphoblastoid cell lines (NC1 and NC2) were amplified and digested with *Hha*I. The *LSAMP* promoter (540 bp) contains 28 CpG
25   islands. The analysed 231-bp fragment of the *LSAMP* promoter contains one *Hha*I site and digestion leads to fragments of 162 bp and 69 bp. Representative aberrant methylation of the *LSAMP* promoter in sporadic and familial CC-RCC samples and in RCC cell lines are shown.

Fig. 3C shows methylation analysis of the *NORE1A* promoter by restriction digestion with *Taq*I in the same cohort of samples. The examined 335 bp of the promoter contains 35
30   CpG sequences. The methylated fragment contains two *Taq*I sites and digestion results in bands of 202, 123, and 10 bp. The sizes of molecular weight markers (M) are shown on the left. N, normal kidney sample; T, RCC.

Figure 4 shows suppression of LSAMP, NORE1A, and Nore1 re-expression on cell proliferation characteristics.

Fig. 4A shows re-expression and localization of EGFP-LSAMP, -NORE1A, and -Nore1 fusion protein 2 h after microinjection or 24 h after lipid-mediated transfection of *pEGFP-*

5   *LSAMP, -NORE1A,* and *-Nore1* plasmids.

Fig. 42B shows a growth inhibition assay. A-498/Caki-1 cells were microinjected with *pEGFP-LSAMP, -NORE1A, -Nore1,* or *pEGFP-C1/-N1* vector (negative control). Cell proliferation analysis was performed 2 h after microinjection. Cells were counted at the indicated times. The "proliferation index" on the y-axis represents the number of cells counted

10   at those times divided by the number of cells counted 2 h after injection.

Figure 5 and its subparts show cytogenetic analysis of the t(1q32.1;3q13.3). **Fig. 5A** shows G-banding and spectral karyotyping analysis. The breakpoints were marked with arrows.

Fig. 5B shows representative results of FISH with the BAC clone probes on t(1;3) breakpoint region. **Fig. 5B-1** (Left panel) shows FISH with BAC probe CTD-2321B11 (red

15   signal): a split signal was observed in der(3). **Fig. 5B-2** (Right panel) shows FISH with probe RP11-281N16: split signals (red) equally appeared on both der(1) and der(3). A 1q subtelomeric PAC probe 160H23 (green signal) was used as a control in all the FISH experiments.

Figure 6 and its subparts show two *NORE1* alterations identified in sporadic tumors T31 and T24. **Fig. 6A** shows that the codon 189 GTG(Val) was replaced by ATG(Met) in T31.

20   It was also found in 5% of the control chromosomes tested. **Fig. 6B** shows that the codon 248 CGG(Arg) was replaced by CAG(Gln) in T24, which has not been detected in control samples. In *NORE1B,* the affected codon number is 95.

Figure 7 shows nuclear localization and growth suppression analysis of Nore1. **Fig. 7A** demonstrates that EGFP-Nore1 is predominantly nuclear in Caki-1 cells (RCC cell line), by

25   lipid-mediated transfection using a *pEGFP-Nore1* plasmid and LIPOFECTAMINE 2000 reagent (Invitrogen). EGFP expressed from the empty vector *pEGFP-C1* was both nuclear and cytoplasmic. **Fig. 7B** shows that subcellular fractionation and subsequent Western analysis further indicated that the majority of Nore1 is localized to the nucleus, while a lesser amount appears at the plasma membrane. **Fig. 7C** shows that induction of Nore1 inhibits the growth of

30   293-T cells. The 293-T cells were transfected with *pIND(SP1)-Nore1* or an empty vector and selected in hygromycin for three weeks. Each point on the growth curve represents the mean of

three individual cell count determinations. **Fig. 7D** shows western analysis of Nore1 expression; V, vector-transfected cells; M, Nore1/MaxP1-transfected cells.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The inventors demonstrate here that the limbic-system-associated membrane protein
5   (*LSAMP*) gene and the *NORE1* gene are the breakpoint-spanning genes in a familial clear cell renal cell carcinoma (CC-RCC), and that the expression of these genes is down-regulated in RCC cell lines and sporadic CC-RCCs. Furthermore, expression of LSAMP and NORE1A proteins in CC-RCC cell lines is shown to inhibit cell proliferation. Diagnostic and treatment methods based on the above observations are described below.

10   This invention includes a method for detecting the presence of, or a predisposition (susceptibility) to, a cancer (*e.g.*, CC-RCC) in a subject, comprising detecting, measuring the amount of, or quantitating LSAMP and/or NORE1 gene expression in a sample from the subject, compared to a baseline level of expression, wherein a reduction in the expression of one or both of the genes compared to the baseline level indicates that the subject suffers from, or has a
15   predisposition to, the cancer.

As used herein, a "baseline value" or "baseline amount" includes the amount of expression of an LSAMP or a NORE1 gene in normal tissue, such as from a "pool" of normal subjects who do not suffer from, or who do not exhibit a predisposition to, the cancer. This value can be determined at the same time as the level in a sample from the subject being studied,
20   or it can be available in a reference database such as a reference standard or a generic database.

The expression may be at the level of RNA transcription which can be detected by various means including quantitative hybridization to a suitable probe, or at the level of protein translation, for example by determining the activity of, or the presence of, the protein, using conventional procedures including an immunoassay. Methods for detecting, measuring or
25   quantitating either the RNA or the protein gene product are conventional and routine.

In the following description, reference will be made to various methodologies known to those of skill in the art of immunology, cell biology, and molecular biology. Publications and other materials setting forth such known methodologies to which reference is made are incorporated herein by reference in their entireties as though set forth in full. Standard reference
30   works setting forth the general principles of immunology include A.K. Abbas *et al.*, *Cellular and Molecular Immunology* (Fourth Ed.), W.B. Saunders Co., Philadelphia, 2000; C.A. Janeway

et al., Immunobiology. The Immune System in Health and Disease, Fourth ed., Garland
Publishing Co., New York, 1999; Roitt, I. *et al., Immunology*, (current ed.) C.V. Mosby Co., St.
Louis, MO (1999); Klein, J., *Immunology*, Blackwell Scientific Publications, Inc., Cambridge,
MA, (1990).

5   Monoclonal antibodies (mAbs) and methods for their production and use are described in
Kohler and Milstein, *Nature 256*:495-497 (1975); U.S. Patent No. 4,376,110; Harlow, E. *et al.,
Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor,
NY, 1988); *Monoclonal Antibodies and Hybridomas: A New Dimension in Biological Analyses*,
Plenum Press, New York, NY (1980); H. Zola *et al.*, in *Monoclonal Hybridoma Antibodies:*

10   *Techniques and Applications,* CRC Press, 1982)); (Kozbor *et al.*, 1983, *Immunol. Today 4*:72 (the
human B-cell hybridoma technique), and Cole, *et al.*, 1985, In: *Monoclonal Antibodies and Cancer
Therapy*, Alan R. Liss, Inc., pp. 77-96 (the EBV-hybridoma technique to produce human mAbs).

Interspecies chimeric antibodies are described, for example, in Cabilly *et al.*, U.S.
Patents 4,816,567 (3/28/89) and 6,331,415 (12/18/01);; Morrison *et al.*, US Patent 5,807,715

15   (9/15/98) and Eur. Patent Pub. EP173494 (3/5/86); Taniguchi *et al.*, Eur. Patent Pub. EP171496
(2/19/86); Neuberger *et al.*, PCT Pub. WO86/01533 (3/13/86); Robinson *et al.*, PCT Pub. WO
8702671 (5/7/87); Cabilly *et al.*, *Proc. Natl. Acad. Sci. USA 81*:3273-3277 (1984); Morrison *et
al.*, *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984); Boulianne *et al.*, *Nature 312*:643-646
(1984); Morrison, *Science, 229*:1202-1207 (1985); Neuberger *et al., Nature 314*:268-270

20   (1985); Takeda *et al., Nature 314*:452-454 (1985); Oi *et al., BioTechniques 4*:214 (1986); Sun *et
al., Proc. Natl. Acad. Sci. USA 84*:214-218 (1987); Liu *et al., J. Immunol. 139*:3521-3526
(1987); Better, M., *et al., Science 240*:1041-1043 (May 20, 1988); and Horwitz, A. H., *et al.,
Proc. Natl. Acad. Sci. USA 85*:8676-8682 (1988)).

Single chain antibodies (scFv) are described, for example, in Skerra, A. *et al.* (1988)

25   *Science*, 240: 1038-1041; Pluckthun, A. *et al.* (1989) *Methods Enzymol.* 178: 497-515; Winter,
G. *et al.* (1991) *Nature*, 349: 293-299); Bird *et al.*, (1988) *Science* 242:423; Huston *et al.* (1988)
Proc. Natl. Acad. Sci. USA 85:5879; Jost CR *et al,. J Biol Chem.* 1994 *269*:26267-26273.U.S.
Patents No. 4,704,692, 4,853,871, 4,946,778, 5,260,203, 5,455,030; and Jost CR *et al. J Biol
Chem.* 1994 *269*:26267-26273.

30   Immunoassay methods are also described in Coligan, J.E. *et al.*, eds., *Current Protocols
in Immunology*, Sec. 2.4.1, Wiley-Interscience, New York, 1992 or current edition); Butt, W.R.
(ed.) *Practical Immunoassay: The State of the Art*, Dekker, New York, 1984; Bizollon, Ch. A.,

ed., *Monoclonal Antibodies and New Trends in Immunoassays*, Elsevier, New York, 1984; Butler, J.E., ELISA (Chapter 29), In: van Oss, C.J. *et al.*, (eds), *IMMUNOCHEMISTRY*, Marcel Dekker, Inc., New York, 1994, pp. 759-803; Butler, J.E. (ed.), *Immunochemistry of Solid-Phase Immunoassay*, CRC Press, Boca Raton, 1991; Weintraub, B., *Principles of Radioimmunoassays*,

5   Seventh Training Course on Radioligand Assay Techniques, The Endocrine Society, March, 1986; Work, T.S. *et al.*, *Laboratory Techniques and Biochemistry in Molecular Biology*, North Holland Publishing Company, NY, (1978) (Chapter by Chard, T., "An Introduction to Radioimmune Assay and Related Techniques").

      In a preferred embodiment, the presence or amount of LSAMP and/or NORE1 protein in

10   a cell is detected by binding proteins in the sample to a detectably labeled antibody that is specific for an LSAMP or a NORE1 protein. An antibody "specific" for a polypeptide means that the antibody recognizes a defined sequence of amino acids, or epitope, either present in the full length polypeptide, or in a peptide fragment thereof.

      Any of a variety of antibodies can be used in such methods. Such antibodies include,

15   polyclonal, monoclonal (mAbs), recombinant, humanized or partially humanized, single chain (scFv), Fab, and fragments thereof. The antibodies can be of any isotype, such as IgM, various IgG isotypes such as IgG_1' IgG_{2a}, *etc.*, and they can be from any animal species that produces antibodies, including goat, rabbit, mouse, chicken or the like.

      Antibodies are prepared according to conventional methods, which are well known. See,

20   references cited above. Methods of preparing humanized or partially humanized antibodies, and antibody fragments, and methods of purifying antibodies, are conventional (*supra*).

      For preparation of mAbs, any technique that provides mAbs produced by cell lines in continuous culture can be used (*supra*). Techniques described for the production of single chain antibodies (*supra*) can be adapted to produce scFv antibodies to polypeptide products of this

25   invention.

      Transgenic animals may be used to express partially or fully humanized antibodies to immunogenic polypeptide products of this invention.

      Other specific binding partners, such as, *e.g.*, aptamers and peptide nucleic aces (PNA), may be used in place of antibodies.

30   The sample to be assayed in a method of the invention may be any suitable cell or tissue, or extract thereof. A sample of a body fluid such as plasma, serum, urine, saliva, cerebrospinal fluid, etc., may be obtained from the subject being screened. Alternatively, cells expressing the

protein on their surface, *e.g.*, suitable neuronal cells for the detection of LSAMP protein, may be obtained by simple, conventional means. If the protein is a receptor or other cell surface structure, it can be detected and quantified by well-known methods such as flow cytometry, immunofluorescence, immunocytochemistry or immunohistochemistry, or the like (see *supra*).

5   In a preferred embodiment, the detection or diagnosis is performed on a sample from a kidney tumor, *e.g.*, a tissue biopsy, a fresh-frozen sample, or a paraffin-embedded tissue section. Methods of preparing all of these sample types are conventional and well known in the art. Biopsy material and fresh-frozen samples can be extracted by conventional procedures to obtain proteins or polypeptides. In one embodiment, paraffin-embedded blocks are sectioned and

10   analyzed directly without such extraction.

Another embodiment of the invention is a method for inhibiting the growth, transformation or other cancer-associated property of a tumor cell, preferably a CC-RCC cell, which is characterized by reduced expression of the *LSAMP* and/or *NORE1* genes compared to a normal kidney cell or a baseline value. The method comprises contacting the cell with an

15   effective amount of an agent which stimulates the expression of the LSAMP and/or NORE1 polypeptide. By an "effective amount" is meant an amount that leads to a measurable reduction of such expression measured at the RNA or protein level.. Methods of contacting a cell are conventional and include injection or other forms of administration and my be done using liposomes, electroporation, microinjection or the like. The cell may be contacted *in vitro* or *in*

20   *vivo*.

Another embodiment is a method for treating a subject suffering from cancer or a tumor, such as CC-RCC, in which at least some of the cells of the subject under-express the *LSAMP* and/or the *NORE1* gene compared to a baseline value. The method comprises

(1)   administering to the subject an effective amount of LSAMP and/or NORE1 polypeptide

25   or active fragment or variant thereof, or a nucleic acid encoding the polypeptide or active fragment or variant thereof which fragment or variant have the desired biological level of the LSAMP or NORE1 polypeptide; or

(2)   administering an agent which stimulates, promotes or otherwise results in increased expression LSAMP or NORE1 polypeptide.

30   Methods of administering the polypeptide are conventional and include, *e.g.*, systemic administration or, preferentially, direct intratumoral administration. The subject may be any suitable animal, preferably a mammal, more preferably a human.

In the above nucleic acid embodiments, the polynucleotide being administered comprises sequences which encode the polypeptide (or variant or fragment), and which are operably linked to an expression control sequence such as a promoter. This polynucleotide may be cloned in a suitable vector, many examples of which are well known to those of skill in the art.

5   As used herein, the term "expression control sequence" means a polynucleotide sequence that regulates expression of a polypeptide encoded by a polynucleotide to which the control sequence is functionally ("operably") linked. Expression can be regulated at the level of the mRNA or polypeptide synthesis or stability. Thus, the "term expression control sequence" includes mRNA-related elements and protein-related elements, which include promoters,

10   domains within promoters, upstream elements, enhancers, elements that confer tissue or cell specificity, response elements, ribosome binding sequences, transcriptional terminators, *etc.* An expression control sequence is operably linked to a nucleotide sequence (*e.g.*, a coding sequence) when the expression control sequence is positioned in such a manner to effect or achieve expression of the coding sequence. For example, when a promoter is operably linked 5'

15   to a coding sequence, expression of the coding sequence is driven by the promoter. Suitable expression control sequences will be evident to the skilled worker.

Methods for generating polynucleotides and polypeptides for use in the methods, compositions and kits of the invention, are conventional. For example, polynucleotides can be isolated, *e.g.*, using sequence probes corresponding to the sequences indicated in the GenBank

20   accession numbers provided elsewhere herein. The polynucleotides can be cloned into suitable vectors, and introduced into and replicated and/or expressed in suitable host cells. Procedures for carrying out these steps are conventional. Nucleic acids that have replicated in the cells, and polypeptides expressed in the cells, can be harvested and, if desired, purified, using conventional procedures. Some suitable molecular biology methods, for use in these and other aspects of the

25   invention, are provided *e.g.*, in Sambrook, *et al.* (1989), *Molecular Cloning, a Laboratory Manual*, Cold Harbor Laboratory Press, Cold Spring Harbor, N.Y.; Ausubel *et al.* (1995). *Current Protocols in Molecular Biology*, N.Y., John Wiley & Sons; Davis *et al.* (1986), *Basic Methods in Molecular Biology*, Elsevier Sciences Publishing,, Inc., New York; Hames *et al.* (1985), *Nucleic Acid Hybridization*, IL Press; Dracopoli *et al. Current Protocols in Human*

30   *Genetics*, John Wiley & Sons, Inc.; and Coligan *et al. Current Protocols in Protein Science*, John Wiley & Sons, Inc.

Methods for providing a polynucleotide to a cell *in vitro, i.e.,* contacting the cell, are conventional and include, transfection, a gene gun, microinjection, electroporation, introduction by liposomes or with viral or non-viral vectors, *etc.*

For gene gun-mediated DNA injection, DNA-coated gold particles (*e.g.,* about 1 µg

5   DNA/bullet) are delivered using a helium-driven gene gun (BioRad, Hercules, CA) with a discharge pressure of, for example, about 400 p.s.i. The Biojector 2000 (Bioject Inc., Portland, OR) is a needle-free jet injection device consisting of an injector and a disposable syringe. The orifice size controls the depth of penetration. For example, DNA (at between about 1 and 100 µg) may be delivered using the Biojector with a syringe nozzle. This may be done intradermally,

10   intramuscularly or intratumorally. Follow-up injections using both methods can be repeated as needed, *e.g.,* at weekly intervals.

Methods of gene therapy or nucleic acid therapy, in which a polynucleotide of the invention is provided in a delivery vehicle, are well-known. The gene vehicle may be of viral or non-viral origin (see generally, Jolly, *Cancer Gene Therapy* 1:51-64 (1994) Kimura, *Human*

15   *Gene Therapy* 5:845-852 (1994); Connelly, *Human Gene Therapy* 1:185-193 (1995); and Kaplitt, *Nature Genetics* 6:148-153 (1994). Vehicles for delivery of nucleic acid constructs including a coding sequence of a therapeutic embodiment of the invention can be administered either locally or systemically. These constructs can utilize viral or non-viral vector approaches. Expression of the coding sequences can be induced using endogenous mammalian or

20   heterologous promoters. Expression of the coding sequence can be either constitutive or regulated.

Recombinant retroviruses constructed to carry or express a selected nucleic acid molecule of interest may be used. See, for example, EP 0415731; WO 90/07936; WO 94/03622; WO 93/25698; WO 93/25234; U.S. Patent No. 5,219,740; WO 93/11230; WO

25   93/10218; Vile *et al.,*, *Canc Res* 53:3860-3864 (1993); Vile *et al., Canc. Res.* 53:962-967 (1993); Ram *et al., Canc. Res.* 53:83-88 (1993); Takamiya *et al., J. Neurosci. Res.* 33:493-503 (1992); Baba *et al., J. Neurosurg.* 79:729-735 (1993); U.S. Patent No. 4,777,127; GB Patent No. 2,200,651; and EP 0345242. Preferred recombinant retroviruses include those described in WO 91/02805.

30   Packaging cell lines suitable for use with the above-described retroviral vector constructs may be readily prepared (WO 95/30763 and WO 92/05266), and used to create producer cell lines (also termed vector cell lines) for the production of recombinant vector particles. Preferred

embodiments of the invention utilize packaging cell lines made from human (such as HT1080 cells) or from mink parent cell lines, that result in production of recombinant retroviruses that survive inactivation in human serum.

Alphavirus-based vectors can function as gene delivery vehicles and be constructed from a wide variety of alphaviruses, including, for example, Sindbis virus vectors, Semliki forest virus (ATCC VR-67; ATCC VR-1247), Ross River virus (ATCC VR-373; ATCC VR-1246) and Venezuelan equine encephalitis virus (ATCC VR-923; ATCC VR-1250 ATCC VR-1249; ATCC VR-532). Representative examples of such vector systems are described in U.S. Patents No. 5,091,309; 5,217,879; and 5,185,440; and PCT Publications WO 92/10578; WO 94/21792; WO 95/27069; WO 95/27044; and WO 95/07994.

Delivery vehicles of the present invention can also employ parvovirus such as adeno-associated virus (AAV) vectors. See, for example, Srivastava, WO 93/09239, Samulski *et al., J. Vir.* 63:3822-3828 (1989); Mendelson *et al., Virol.* 166:154-165 (1988); and Flotte *et al., P.N.A.S.* 90:10613-10617 (1993).

Representative examples of adenoviral vectors are described by Berkner, *Biotechniques* 6:616-627; Rosenfeld *et al., Science* 252:431-434 (1991); WO 93/19191; Kolls *et al., Proc. Natl. Acad Sci. USA.* 91:215-219 (1994); Kass-Eisler *et al., Proc. Natl. Acad Sci. USA* 90:11498-11502 (1993); Guzman *et al., Circulation* 88:2838-2848 (1993); Guzman *et al., Cir. Res.* 73:1202-1207 (1993); Zabner *et al., Cell* 75:207-216 (1993); Li *et al., Hum. Gene Ther.* 4:403-409 (1993); Cailaud *et al., Eur. J. Neurosci.* 5: 1287-1291 (1993); Vincent *et al., Nat. Genet.* 5:130-134 (1993); Jaffe *et al., Nat. Genet.* 1:372-378 (1992); and Levrero *et al., Gene* 101:195-202 (1992). Exemplary adenoviral nucleic acid therapy vectors useful herein are described in WO 94/12649, WO 93/03769; WO 93/19191; WO 94/28938; WO 95/11984 and WO 95/00655. Administration of DNA linked to killed adenovirus is described in Curiel, *Hum. Gene Ther.* 3:147-154 (1992).

Other delivery vehicles and methods may be employed, including polycationic condensed DNA linked or unlinked to killed adenovirus alone, for example, Curiel (*supra*); ligand-linked DNA, for example, see Wu, *J. Biol. Chem.* 264:16985-16987 (1989); eukaryotic cell delivery vehicles (U.S.S.N. 08/240,030, filed May 9, 1994, and 08/404,796); deposition of photopolymerized hydrogel materials; hand-held gene transfer particle gun (U.S. Patent 5,149,655); ionizing radiation (U.S. Patent No. 5,206,152 and WO 92/11033; nucleic acid charge neutralization or fusion with cell membranes. Additional approaches are described in

Philip, *Mol. Cell Biol.* 14:2411-2418 (1994) and Woffendin, *Proc. Natl. Acad. Sci. USA*
91:1581-1585 (1994) (a mechanical delivery system).

 Naked DNA may also be employed. See, for example, WO 90/11092 and U.S. Patent
5,580,859. Uptake efficiency may be improved using biodegradable latex beads. DNA coated
5 latex beads are efficiently transported into cells after endocytosis initiation by beads. The
method may be improved further by treatment of the beads to increase hydrophobicity and
thereby facilitate disruption of the endosome and release of the DNA into thr cytoplasm.

 Use of liposomes as DNA delivery vehicles are described in U.S. Patent 5,422,120, PCT
Patent Pub. WO 95/13796, WO 94/23697 and WO 91/14445, and EP 0 524 968.

10 Effective dosages and routes of administration of polypeptides or polynucleotides of the
invention are conventional. The exact amount (effective dose) of the agent will vary from
subject to subject, depending on the species, age, weight and general or clinical condition of the
subject, the severity or mechanism of any disorder being treated, the particular agent or vehicle
used, the method and scheduling of administration, and the like. A therapeutically effective
15 dose can be determined empirically, by conventional procedures known to those of skill in the
art. See, *e.g.*, *The Pharmacological Basis of Therapeutics*, Goodman and Gilman, eds.,
Macmillan Publishing Co., New York. For example, an effective dose can be estimated
initially either in cell culture assays or in suitable animal models. The animal model may also
be used to determine the appropriate concentration ranges and routes of administration. Such
20 information can then be used to determine useful doses and routes for administration in
humans. A therapeutic dose can also be selected by analogy to dosages for comparable
therapeutic agents. In general, effective doses include between about 10 ng to about 100 mg up
to a total dose of about 5g, depending on the route of administration, number of repeat
administrations and other factors as noted above.

25 A variety of routes of administration may be used, including oral, respiratory, intranasal,
intrarectal, intravaginal, sublingual, transdermal, extracorporeal, topical, intravenous,
subcutaneous, intramuscular, intramedullary, or intraperitoneal injection, other parenteral
routes, or the like. One of skill in the art will recognize particular cells, tissues or organs into
which therapeutic agents of the invention can be administered, as appropriate for particular
30 indications.

 Another embodiment of the invention is a pharmaceutical composition comprising (a) an
LSAMP and/or NORE1 polypeptide, or an active fragment or variant thereof, or (b) a

polynucleotide encoding an LSAMP and/or NORE1 polypeptide, or encoding an active fragment or variant of the polypeptide, wherein the polynucleotide is operably linked to an expression control sequence; and a pharmaceutically acceptable carrier.

Another embodiment of the invention is a kit, suitable for carrying out any method of the

5   invention. For example, the invention includes a kit for detecting the presence and/or amount of an LSAMP and/or a NORE1 polypeptide in a tumor or pre-cancerous sample, such as CC-RCC or normal kidney cells susceptible of transformation to become CC-RCC, wherein the cells are characterized by a reduced amount compared to a baseline value of one or both of these polypeptides. The kit comprises one or more reagents for detecting the polypeptide, preferably

10   an antibody specific for the polypeptide, which is preferably detectably labeled, and, optionally, one or more reagents for testing the binding of the antibody to a sample polypeptide and/or that one that facilitates detection of antibody binding.

Another embodiment is a kit for detecting the presence and/or amount of a polynucleotide encoding LSAMP and/or NORE1 polypeptide in a tumor or pre-cancerous

15   sample, such as a CC-RCC tumor cells or normal kidney cells susceptible of transformation to become CC-RCC. Such cells are characterized by under-expression of LSAMP and/or NORE1 compared to a baseline value which is indicative that the cells are susceptible of development into cancer cells, primarily CC-RCC cells. The kit comprises a nucleic acid probe specific for a LSAMP- or NORE1-encoding polynucleotide, and, optionally, one or more reagents that

20   facilitate hybridization of the probe to the sample polynucleotide, and/or that facilitate detection of the hybridized polynucleotide.

Another embodiment is a kit useful for inhibiting or reducing a cancer-associated property of a cell, comprising an LSAMP and/or NORE1 polypeptide, or an active fragment or variant thereof, and, optionally, means for introducing the polypeptide into the cell and/or for measuring

25   the cancer-associated property.

In another embodiment, the kit is suitable for treating a subject suffering from a cancer such as CC-RCC, the kit comprising an LSAMP and/or NORE1 polypeptide, or an active fragment or variant thereof, and, optionally, means for administering the polypeptide to the subject.

30   In other embodiments, the kit comprises, instead of the polypeptides, a nucleic acid encoding LSAMP and/or NORE1, or encoding active fragments or variants of the polypeptides, wherein the polynucleotides are operably linked to expression control sequences.

14

Optionally, kits of the invention comprise instructions for performing the method for which the kit is intended and/or for analyzing and/or evaluating the assay results as generated by the method. A kit may further comprise a support on which a cell can be propagated (*e.g.*, a tissue culture vessel) or a support to which a reagent used in the method is immobilized. Other

5   optional elements of the present kit include suitable buffers, media components, or the like; reagents for performing suitable controls; a computer or computer-readable medium for storing and/or evaluating the assay results; containers; or packaging materials. The reagents of the kit may be in containers in which the reagents are stable, *e.g.*, in lyophilized form or stabilized liquids. The reagents may also be in single use form, *e.g.*, in single dosage form for use as

10   therapeutics, or in single reaction form for diagnostic use.

Another embodiment of the invention is an antibody specific for an epitope of the LSAMP or he NORE1 polypeptide. Such antibodies are useful, not only for diagnostic procedures, as discussed above, but also for experimental purposes, *e.g.*, for elucidating the mechanisms of CC-RCC carcinogenesis. Some of the types of suitable antibodies, and methods

15   for using them, were discussed above.

EXAMPLES

EXAMPLE I

Mapping of Breakpoints

The inventors have previously mapped the constitutional t(1;3)-associated breakpoints to

20   bands 1q32.1 and 3q13.3 in a family with four cases of CC-RCC (Kanayama *et al.*, 2001). In this study, the inventors cloned the breakpoints of t(1;3)(q32.1;q13.3) by using a strategy that combined FISH, Southern blot, long-range PCR, and DNA sequencing. FISH experiments allowed narrowing of the breakpoint regions to a 20- to 30-kb range on both affected chromosomes (Figure 1A). These were further refined via Southern blot analyses and restriction

25   mapping to approximately 2 kb (Figure 1B and 1C). Assisted by information from human genome sequence databases and BAC clone databases, several sets of specific primers were designed around the breakpoints and long-range PCR was performed to amplify the breakpoint fragments (Figure 2A). A 2.15 kb der(1)-breakpoint and a 3.25 kb der(3)-breakpoint were amplified and subcloned into TA-cloning vector (Invitrogen, USA) (Figure 2A). Subsequent

30   DNA sequencing of the breakpoint fragments resulted in the identification of both breakpoints (Figure 2; also see Table 1).

15

Table 1.
Sequences and positions of the synthetic oligonucleotides used in the study.

Target	Direction	Sequence (5'-3')	SEQ ID NO:
For genomic PCR of the fusion genes			
NORE1-LSAMP	Forward	GGAGAAAGAGAGACCAGGACAAA (NORE1)	1
NORE1-LSAMP	Reverse	GCTTCCCAGGTTCAAGTGATTC (LSAMP)	2
NORE1-LSAMP	Sequencing Forward	AGCGATCATCCTGCCTTG (NORE1)	3
NORE1-LSAMP	Sequencing Forward	CATGCCAGGCCCATAAATAG (NORE1)	4
NORE1-LSAMP	Sequencing Forward	GTGGCCTGCAAAACCTAAC (NORE1)	5
LSAMP-NORE1A	Forward	AATCCAGGTCTCTCTGCTCCAA (LSAMP)	6
LSAMP-NORE1A	Reverse	CTCTGAGTGAGTCACGTGGCTT (NORE1)	7
LSAMP-NORE1A	Forward	CCTTCCTTTTCTCCATAGCACT (LSAMP)	8
LSAMP-NORE1A	Forward	ATTGTGGAAAATGGAGCTTC (LSAMP)	9
For fusion transcript analyses			
NORE1A-LSAMP	Forward	AGTCAGCAGGAGGGTTTATCC (NORE1A)	10
NORE1A-LSAMP	Reverse	CCCTTCCAGTTGGTGTAAGGT (LSAMP)	11
LSAMP-NORE1A	Forward	TTCTCTGGAATACAGCCTCCG (LSAMP)	12
LSAMP-NORE1A	Reverse	GCGTGTTGTAGCTGTCGATC (NORE1A)	13
NORE1B-LSAMP	Forward	GCAGCATGAGCAGTGGGTAC (NORE1B)	14
NORE1B-LSAMP	Reverse	TTTGCCTGACTGCTCCCTG (LSAMP)	15
For mutation analyses			
NORE1gene			
NORE1A Exon 1	Forward	TCCTTCCTGCCACTCCGACTC	16
NORE1A Exon 1	Reverse	TCCCAAGAACTCACAACAAAACC	17
NORE1A Exon 1	Sequencing Forward	TCCTTCCTGCCACTCCGACT	18
NORE1A Exon 1	Sequencing Reverse	TCCTCGCGCCTCTGTGTCCC	19
NORE1A Exon 2	Forward	TCCAAGGTTATTTCTCTGGGTG	20
NORE1A Exon 2	Reverse	GAGTTCTCTGTGTCACTTCCCC	21
NORE1A Exon 3	Forward	CTGGATGCTCACTTCTTGGTTAG	22
NORE1A Exon 3	Reverse	CAGAATTCAGAGTGAGGGCAG	23
NORE1A Exon 4	Forward	AGAACTCAAGGAGACAGGTGGG	24
			25

Target	Direction	Sequence (5′-3′)	SEQ ID NO:
NORE1A Exon 4	Reverse	AGATCTGAACACCACATGGGC	26
NORE1A Exon 5	Forward	CACCTCTGCATTTCCAATCCTT	27
NORE1A Exon 5	Reverse	GTGGCTCCCACCTATGTGAG	28
NORE1A Exon 6	Forward	CAGGGTCTCTCAGGTCGTGTCA	29
NORE1A Exon 6	Reverse	CCCCCATGCAAACACTTGTC	30
NORE1B Exon1	Forward	CCCGCTGAAAGAAACGCAGG	31
NORE1B Exon1	Reverse	ATGCTCAGCCCTCAGGGCAA	32
***LSAMP*gene**			
LSAMP Exon1	Forward	AGTGGAAAGGACCATAAACTGGC	33
LSAMP Exon1	Reverse	TGGAGTTCAAGGAGATCAGACAC	34
LSAMP Exon2	Forward	ATGACATCCATCCACTGGATG	35
LSAMP Exon2	Reverse	TGCAACTCCCACCTCTTTCTTA	36
LSAMP Exon3	Forward	AGATGGCAAGCATGGGTCTTA	37
LSAMP Exon3	Reverse	TCAGCAGAATTCCAGGAGCA	38
LSAMP Exon4	Forward	CTGCTTCTGTGGAATCTGATGTC	39
LSAMP Exon4	Reverse	CAAAGACCAAGTCCTGCCCTT	40
LSAMP Exon5	Forward	CTCCCTTCCTGCCTCTCTCTAA	41
LSAMP Exon5	Reverse	GCTTAAGAGCTACAGGCCCC	42
LSAMP Exon6	Forward	TCCTTTTCCTCCAGTGTCAGG	43
LSAMP Exon6	Reverse	TGCTATGCACAGGAGTTGAGAA	44
LSAMP Exon7	Forward	CTTCTTGGGCTGCACATAAGTG	45
LSAMP Exon7	Reverse	ACGGTCTCCCCCATCTCTCT	46
***VHL* gene**			
VHL Exon1	Forward	TGTAAAACGACGGCCAGTCGAAGAGTACGGCCCTGAAGAAGAC	47
VHL Exon1	Reverse	CAGGAAACAGCTATGACCCAGTACCCTGGATGTGTCCTGCCTC	48
VHL Exon2	Forward	TGTAAAACGACGGCCAGTAGACGAGGTTTCACCACGTTAGC	49
VHL Exon2	Reverse	CAGGAAACAGCTATGACCGTCCTCTATCCTGTACTTACCAC	50
VHL Exon3	Forward	TGTAAAACGACGGCCAGTCTGAGACCCTAGTCTGCCACTGAGGAT	51
VHL Exon3	Reverse	CAGGAAACAGCTATGACCCAAAAGCTGAGATGAAACAGTGTAAGT	52

17

Target	Direction	Sequence (5′-3′)	SEQ ID NO:
For genomic PCR of the fusion genes			
NORE1-LSAMP	Forward	GGAGAAAGAGAGACCAGGACAAA (*NORE1*)	53
NORE1-LSAMP	Reverse	GCTTCCCAGGTTCAAGTGATTC (*LSAMP*)	54
NORE1-LSAMP	Sequencing Forward	AGCGATCATCCTGCCTTG (*NORE1*)	55
NORE1-LSAMP	Sequencing Forward	CATGCCAGGCCCATAAATAG (*NORE1*)	56
NORE1-LSAMP	Sequencing Forward	GTGGCCTGCAAAACCTAAC (*NORE1*)	57
LSAMP-NORE1A	Forward	AATCCAGGTCTCTCTGCTCCAA (*LSAMP*)	58
LSAMP-NORE1A	Reverse	CTCTGAGTGAGTCACGTGGCTT (*NORE1*)	59
LSAMP-NORE1A	Forward	CCTTCCTTTTCTCCATAGCACT (*LSAMP*)	60
LSAMP-NORE1A	Forward	ATTGTGGAAAATGGAGCTTC (*LSAMP*)	61
For fusion transcript analyses			
NORE1A-LSAMP	Forward	AGTCAGCAGGAGGGTTTATCC (*NORE1A*)	62
NORE1A-LSAMP	Reverse	CCCTTCCAGTTGGTGTAAGGT (*LSAMP*)	63
LSAMP-NORE1A	Forward	TTCTCTGGAATACAGCCTCCG (*LSAMP*)	64
LSAMP-NORE1A	Reverse	GCGTGTTGTAGCTGTCGATC (*NORE1A*)	65
NORE1B-LSAMP	Forward	GCAGCATGAGCAGTGGGTAC (*NORE1B*)	66
NORE1B-LSAMP	Reverse	TTTGCCTGACTGCTCCCTG (*LSAMP*)	67
For mutation analyses			
NORE1gene			
NORE1A Exon 1	Forward	TCCTTCCTGCCACTCCGACTC	69
NORE1A Exon 1	Reverse	TCCCAAGAACTCACAACAAAACC	71
NORE1A Exon 1	Sequencing Forward	TCCTTCCTGCCACTCCGACT	72
NORE1A Exon 1	Sequencing Reverse	TCCTCGCGCCTCTGTGTCCC	73
NORE1A Exon 2	Forward	TCCAAGGTTATTTCTCTGGGTG	74
NORE1A Exon 2	Reverse	GAGTTCTCTGTGTCACTTCCCC	75
NORE1A Exon 3	Forward	CTGGATGCTCACTTCTTGGTTAG	76
NORE1A Exon 3	Reverse	CAGAATTCAGAGTGAGGGCAG	77
NORE1A Exon 4	Forward	AGAACTCAAGGAGACAGGTGGG	78
NORE1A Exon 4	Reverse	AGATCTGAACACCACATGGGC	79

Target	Direction	Sequence (5′-3′)	SEQ ID NO:
NORE1A Exon 5	Forward	CACCTCTGCATTTCCAATCCTT	80
NORE1A Exon 5	Reverse	GTGGCTCCCACCTATGTGAG	81
NORE1A Exon 6	Forward	CAGGGTCTCTCAGGTCGTGTCA	82
NORE1A Exon 6	Reverse	CCCCCATGCAAACACTTGTC	83
NORE1B Exon1	Forward	CCCGCTGAAAGAAACGCAGG	84
NORE1B Exon1	Reverse	ATGCTCAGCCCTCAGGGCAA	85
LSAMPgene			
LSAMP Exon1	Forward	AGTGGAAAGGACCATAAACTGGC	86
LSAMP Exon1	Reverse	TGGAGTTCAAGGAGATCAGACAC	87
LSAMP Exon2	Forward	ATGACATCCATCCACTGGATG	88
LSAMP Exon2	Reverse	TGCAACTCCCACCTCTTTCTTA	89
LSAMP Exon3	Forward	AGATGGCAAGCATGGGTCTTA	90
LSAMP Exon3	Reverse	TCAGCAGAATTCCAGGAGCA	91
LSAMP Exon4	Forward	CTGCTTCTGTGGAATCTGATGTC	92
LSAMP Exon4	Reverse	CAAAGACCAAGTCCTGCCCTT	93
LSAMP Exon5	Forward	CTCCCTTCCTGCCTCTCTCTAA	94
LSAMP Exon5	Reverse	GCTTAAGAGCTACAGGCCCC	95
LSAMP Exon6	Forward	TCCTTTTCCTCCAGTGTCAGG	96
LSAMP Exon6	Reverse	TGCTATGCACAGGAGTTGAGAA	97
LSAMP Exon7	Forward	CTTCTTGGGCTGCACATAAGTG	98
LSAMP Exon7	Reverse	ACGGTCTCCCCCATCTCTCT	99
VHL gene			
VHL Exon1	Forward	TGTAAAACGACGGCCAGTCGAAGAGTACGGCCCTGAAGAAGAC	100
VHL Exon1	Reverse	CAGGAAACAGCTATGACCCAGTACCCTGGATGTGTCCTGCCTC	101
VHL Exon2	Forward	TGTAAAACGACGGCCAGTAGACGAGGTTTCACCACGTTAGC	102
VHL Exon2	Reverse	CAGGAAACAGCTATGACCGTCCTCTATCCTGTACTTACCAC	103
VHL Exon3	Forward	TGTAAAACGACGGCCAGTCTGAGACCCTAGTCTGCCACTGAGGAT	104
VHL Exon3	Reverse	CAGGAAACAGCTATGACCCAAAAGCTGAGATGAAACAGTGTAAGT	105

19

The cloning of the breakpoints led to the identification of two breakpoint-spanning genes, *NORE1* on 1q32.1 and *LSAMP* on 3q13.3 (Figure 2B). To investigate whether fusion proteins resulting from the chromosome translocation are involved in tumorigenesis of CC-RCC, Northern blot analysis and RT-PCR were carried out to detect any fusion transcript of

5   *NORE1* and *LSAMP* (see Table A in supplemental data). No detectable fusion transcripts were found in the FRCC3 and FRCC5 cell lines from two patients in the t(1;3) family. The possible sequence combination from *NORE1* and *LSAMP* were also tested. Since *NORE1* lies in the positive DNA strand and *LSAMP* in the reverse strand, there is little likelihood for them to form any *NORE1-LSAMP* or *LSAMP-NORE1* fusion proteins.

10   Given the association between chromosome 3 translocations and CC-RCC susceptibility (van Kessel *et al.*, 1999; Bodmer *et al.*, 2002c), the gene *LSAMP* was investigated. *LSAMP* is composed of seven exons and is disrupted in intron 2 by the breakpoint (Figure 2B). To elucidate whether genetic changes in *LSAMP* play a role in CC-RCC, *LSAMP* mutation analysis was performed in 9 CC-RCC cell lines and in 53 sporadic and 4 familial tumors. No *LSAMP*

15   mutation was detected. However, epigenetic silencing in association with hypermethylation, the most common form of inactivation for many tumor suppressor genes (Jones *et al.*, 2002), could still occur. First, RT-PCR analysis showed that LSAMP was down-regulated in all nine RCC cell lines (Figure 3A). Furthermore, the *LSAMP* promoter was methylated in 7/9 CC-RCC cell lines (78%), 14/53 sporadic CC-RCCs (26%), and all 4 familial CC-RCCs tumors from the

20   t(1;3) family (Figure 3B). In association with the promoter-methylation status, LSAMP expression in ten examined tumors with *LSAMP*-promoter methylation was also down-regulated (Figure 3A). Of the *LSAMP*-promoter-methylated cell lines and tumors, all presented complete methylation except two cell lines and one sporadic tumor. Furthermore, in the four familial tumors (FT1 to FT4), one *LSAMP* allele was breakpoint-disrupted followed by the loss of the

25   der(3) chromosome shown in our previous study (Kanayama *et al.*, 2001), and the other copy was hypermethylated (Figure 3B), implying *LSAMP* may undergo bi-allelic inactivation. These observations suggest that *LSAMP* is involved in CC-RCC, though further functional studies are needed to elucidate its mechanism.

The 1q32.1 breakpoint-disrupted gene, *NORE1*, also appeared to be an excellent

30   candidate CC-RCC suppressor gene. *NORE1* undergoes alternative splicing, resulting in two isoforms, *NORE1A* and *NORE1B*. The breakpoint disrupted both *NORE1A* and *NORE1B* (Figure 2B). NORE1 is homologous to a family of RAS binding proteins, including RASSF1,

rat Maxp1, and murine Nore1 (Vavvas *et al.*, 1998; Dammann *et al.*, 2000; Vos *et al.*, 2000; Ortiz-Vega *et al.*, 2002; Tommasi *et al.*, 2002) that have been proposed to be effectors for the small GTPase. Maxp1, Nore1 and RASSF1 have been shown to induce apoptosis (Vos *et al.*, 2000; Khokhlatchev *et al.*, 2002). Other studies, however, have shown that Nore1 family

5   members are cytostatic and modulate cyclinD1 levels, thereby influencing the activity of cell cycle-dependent kinases (Khokhlatchev *et al.*, 2002). *RASSF1* maps to 3p21, a region of frequent LOH in CC-RCC (van den Berg *et al.*, 1997; Dammann *et al.*, 2000), and this gene has recently been shown to be epigenetically inactivated in kidney cancer (Dreijerink *et al.*, 2001; Morrissey *et al.*, 2001; Yoon *et al.*, 2001). Thus, the inventors proceeded to investigate *NORE1*

10   as a candidate RCC suppressor.

Mutation screening and methylation analysis were performed on *NORE1* in all the RCC cell lines and tumors. Two alterations, GTG(Val189)>ATG(Met189) and CGG(Arg248)>CAG(Gln248), were identified (see Figure 6). The former was present in 5% of the 100 tested normal subjects, whereas the latter was not found in any of them. As both were

15   also present in the matched normal kidney tissues, it is likely that they represent polymorphisms. The inventors then perceived that *NORE1A* expression was also down-regulated in the 9 RCC cell lines, and the *NORE1A* promoter was methylated in 6/9 RCC cell lines and 17/53 (32%) sporadic RCC tumors (Figure 3A and 3C), whereas methylation in the *NORE1B* promoter was detected only in RCC cell lines A-498 and A-704. *NORE1A* expression in examined 10 of the 17

20   affected tumors was also down-regulated (Figure 3A). Two normal kidney control samples (N3 and N44) also showed *NORE1A* promoter methylation at lower extents compared with their matched tumors (3T and 44T), probably due to contamination from the tumor tissues. Interestingly, *NORE1A*-promoter methylation does not overlap with *LSAMP*-promoter methylation except in four tumors. These results suggest that *NORE1A* is also associated with

25   sporadic CC-RCC. Yet, unlike the methylation situation in *LSAMP*, only 1/4 hereditary tumors showed even slight *NORE1A* promoter methylation, indicating one wild-type allele of *NORE1A* still exists in these hereditary tumors. Whether *NORE1A* undergoes haploinsufficiency in tumorigenesis remains undetermined.

In addition, 7/14 tumors (50%) with *LSAMP*-promoter methylation showed loss of

30   heterozygosity (LOH) of the *LSAMP* locus. However, LOH was also observed in 17/39 tumors (44%) without *LSAMP*-promoter methylation. Similar LOH results were obtained on *NORE1A*

(methylated, 5/17 [29%]; unmethylated, 8/36, [22%]), indicating that the LOH may be correlated with CC-RCC tumorigenesis, but is not methylation-dependent.

Promoter methylation in both LSAMP and NORE1A may also be linked to other types of cancers. *NORE1A*-promoter methylation has recently been detected in cancer cell lines and

5   in 24% NSCLC (Hesson *et al.*, 2003). Here the inventors found that the *LSAMP* promoter was methylated in 5/19 (26%) colorectal cancers.

The exact role of these genes in tumorigenesis is unclear. Without wishing to be bound to any particular mechanism, potential roles for these genes are discussed below. In the familial cases, the underlying mechanism appears to be the three-step model of chromosome 3

10   translocation-related hereditary CC-RCC tumorigenesis (Bodmer *et al.*, 1998; Kanayama *et al.*, 2001; Bodmer *et al.*, 2002c). Considering the complexity of the multistep process in tumorigenesis, the possibility exists that the breakpoint-disrupted genes, especially *LSAMP*, may contribute to the occurrence of familial tumors by acting as components in the three-step model of tumorigenesis of hereditary CC-RCC. The inventors have previously demonstrated that four

15   examined familial CC-RCC tumors lost the der(3) chromosome and two of them carry *VHL* mutations, supporting the three-step model of tumorigenesis (Kanayama *et al.*, 2001). Here, the inventors supplement this model with our *LSAMP* and *NORE1A* data. The constitutional translocation t(1q;3q) and disruption of a copy each of *LSAMP* and *NORE1*, as the first set of steps of tumorigenesis, act as the predisposing factors in development of CC-RCC. The

20   translocation also results in the increased susceptibility to somatic loss of the chromosome der(3). The following non-disjunctional loss of der(3) deletes a copy each of the RCC-related genes in chromosome 3 (e.g., *VHL*, *RASSF1A*), which further increases the predisposition to CC-RCC. This second set of steps will accelerate the transformation process and cellular growth, leading to the third set of steps involving either the inactivation of the other *VHL* allele

25   (e.g. somatic mutation) or the genetic/epigenetic alternations in other CC-RCC-related genes including *LSAMP* in the remaining copy of chromosome 3. These factors may act synergistically and finally lead to the occurrence of CC-RCC.

Epigenetic inactivation of these genes can be reversed by demethylation treatment with the DNA methylation inhibitor 5-aza-2'-deoxycytidine (5-aza-CdR). The demethylation

30   treatment resulted in significantly increased expression of LSAMP and NORE1 in eight cell lines (Figure 3A), indicating that repression is at least in part mediated by methylation.

Finally, to further evaluate the role of *LSAMP* and *NORE1* as tumor suppressor candidates in cancer, enhanced green fluorescent protein *EGFP-LSAMP*, *-NORE1A*, and *-Nore1* expression plasmids were microinjected or transfected into two RCC cell lines, A-498 and/or Caki-1, in which the *LSAMP* and *NORE1A* promoters were methylated, cells were then counted

5   at indicated times, and were monitored for cell number and/or proliferation. Alternatively, cells were monitored by epi-fluorescence/phase-contrast microscopy to evaluate proliferation, fluorescent protein expression, or apoptosis. While cells expressing EGFP continued to proliferate at rates similar to those of uninjected neighbors, cells expressing EGFP-LSAMP, - NORE1A and -Nore1 failed to divide (Figure 4B). There was no evidence of apoptosis in any

10   of the experiments. This growth inhibition role was also demonstrated in 293-T cells stably transformed with an inducible *Nore1* gene by lipid-mediated transfection (see Figure 7C).

The inventors also observed that EGFP-LSAMP seemed to be cytoplasmic, and EGFP-NORE1A appeared in both cytosol and nucleus. EGFP-Nore1 was predominantly nuclear and tended to occupy discrete puncta within the nucleus (Figure 4A). This was observed in both

15   formaldehyde-fixed and living cells; thus, the localization was unlikely to be due to a fixation artifact. Furthermore, the nuclear localization of EGFP-Nore1 was also confirmed in the transfected Caki-1 RCC cell line and in the 293-T cells by nuclear fractionation (see Figures 7Aa and 7B).

These observations are consistent with a growth suppression role for LSAMP, NORE1A,

20   and NORE1. Also, despite the presence of a putative Ras-association region, the results suggest that this nuclear Nore1 protein may not be a *bona fide* Ras effector, whose family members tend to be lipid-modified, membrane-bound, positive regulators of cell proliferation. Further investigation into its role in growth regulation (potentially through the regulation of cyclin D1 and G1/S progression) and its role in the nucleus are desirable.

25   Based on these data, LSAMP, and NORE1A (a homolog of 3p21-tumor suppressor RASSF1A), represent new tumor suppressor candidates, and presumably act as components in the multistep process of CC-RCC tumorigenesis. Inactivation or reduced expression of both LSAMP and NORE1A also appears to be involved in the occurrence of other types of tumors. Further studies of these genes may lead to the elucidation of novel mechanisms of

30   tumorigenesis.

GenBank accession numbers and Sequences of NORE1A, LSAMP, etc.

NORE1A: GenBank Accession No. NM_031437

Nucleic Acid: SEQ ID NO: 120 (coding: 64-1236); Amino acid: SEQ ID NO:121,

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NORE1B: **GenBank Accession No. AF445801**

Nucleic acid – SEQ ID NO: 122 (coding: 411-1208); Amino acid: SEQ ID NO:123

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TDKRTSFYLP LDAIKQLHIS STTTVSEVIQ GLLKKFMVVD NPQKFALFKR IHKDGQVLFQ
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LSAMP   NM_002338 cDNA

Nucleic acid: SEQ ID NO: 124 (coding: 501-1517); Amino acid SEQ ID NO:125

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1621 aaaaaaaaaa aaaaaaaaaa

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ASSF1A,   GenBank Accession No. AF132675;

Nucleic acid: SEQ ID NO: 126 (coding: 39-1061); Amino acid SEQ ID NO:127

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Norel,   AF053959;

Nucleic acid: SEQ ID NO: 128 (coding: 31-1272); Amin0 Acid: SEQ ID NO: 129

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1141 gaactccaga acttttttaac tatcctggaa aaagaggagc aggacaagat ccatcaactg
1201 caaaagaagt acaacaaatt ccgtcagaaa ctggaagagg cattacgaga gtcccagggg
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1321 aattattttg caacaaagag ttactgttaa gacacctctg gtggttccac cagtcgcctg
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1441 attcctgcta acccaccaca ccatggctct ggagagcttc ccgcctggga tcagaactcc
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1561 ctctctggtc accaaactcc aatcaatcac cagccggcaa aaggaaagaa aggtttcaga
1621 gcctgtgtgt tctttctctg gatttactct tcagttcctc ttttggtttg tttggttggt
1681 tttttttggc cacgtatagt atatttaagg atcaaatgtg gcatattcat tctagctaag
1741 tccttgaaag caggaaaatg ctcatgaaag gactgtcctt gccccaaggt gcctcttctt
1801 ctctagtact agacactcag ggtcagcctg agatttcaag aggctacagc ctgaccaggc
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2041 ctgagctgcc agaaagtatt acagagaagc acatccaagc tgtctgtggc ctacgcctgc
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2281 tgacttccct ggtgacttca gtgcctttt gtttgcagag aaaagagtgg ggcactttgc
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3001 agaaggcatg gctcctgc
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MASPAIGQRP YPLLLDPEPP RYLQSLGGTE PPPPARPRRC IPTALIPAAG ASEDRGGRRS
GRRDPEPTPR DCRHARPVRP GLQPRLRLRP GSHRPRDVRS IFEQPQDPRV LAERGEGHRF
VELALRGGPG WCDLCGREVL RQALRCANCK FTCHSECRSL IQLDCRQKGG PALDRRSPGS
TLTPTLNQNV CKAVEETQHP PTIQEIKQKI DSYNSREKHC LGMKLSEDGT YTGFIKVHLK
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LKKFMVVDNP QKFALFKRIH KDGQVLFQKL SIADYPLYLR LLAGPDTDVL SFVLKENETG
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VHL   GenBank Accession No. NM 000551

Nucleic acids: SEQ ID NO: 129 (coding 2145-855); Amino acids

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   1 cctcgcctcc gttacaacgg cctacggtgc tggaggatcc ttctgcgcac gcgcacagcc
  61 tccggccggc tatttccgcg agcgcgttcc atcctctacc gagcgcgcgc gaagactacg
 121 gaggtcgact cgggagcgcg cacgcagctc cgccccgcgt ccgacccgcg gatcccgcgg
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 601 gttaaccaaa ctgaattatt tgtgccatct ctcaatgttg acggacagcc tattttttgcc
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 961 ttctcagagt aaaataggca ccattgctta aagaaagtt aactgacttc actaggcatt
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2461 tacatagttg agattgtact gttcatacag ttttataccc tttttcattt aactttataa
2521 cttaaatatt gctctatgtt agtataagct tttcacaaac attagtatag tctcccttttt
2581 ataattaatg tttgtgggta tttcttggca tgcatcttta attccttatc ctagcctttg
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2941 accctgcctc aaaaaaaaaa aaaaaaa
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MPRRAENWDE AEVGAEEAGV EEYGPEEDGG EESGAEESGP EESGPEELGA EEEMEAGRPR
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DAGTHDGLLV NQTELFVPSL NVDGQPIFAN ITLPVYTLKE RCLQVVRSLV KPENYRRLDI
VRSLYEDLED HPNVQKDLER LTQERIAHQR MGD

Experimental procedures

Family with CC-RCC and t(1;3)(q32.1;q13.3), paired CC-RCC tumors/normal kidney tissues, and cell lines

The clinical and genetic details of the Japanese kindred with familial CC-RCC have been previously published (Kanayama *et al.*, 2001). The EBV-transformed lymphoblastoid cell lines FRCC3 and FRCC5 used in this study were established from two affected translocation carriers.

Four tumors were from three members of the t(1;3) family, and 53 matched pairs of CC-RCC were collected from the University of Tokushima in Japan.

Nine established RCC cell lines were purchased from ATCC: A-498, A-704, Caki-1, Caki-2, SW-839, ACHN, 786-O, 769-P, and SW-156.

Construction of BAC contigs and FISH analyses

Forty-four 1q32.1 and 3q13.3 BAC clones were obtained from the BACPAC Resource Center (Children's Hospital, Oakland Research Institute) or ResGen Invitrogen Corporation. The clones were selected based on information in the BAC clone mapping databases and Human Genome Sequence Draft database. The details of the BACs are listed in the Supplemental Experimental Procedures.

Standard dual-color FISH was performed by hybridizing each of the 44 BAC clones to metaphase slides prepared from FRCC3 or FRCC5. In all hybridizations, the PAC clone 160H23 from the 1q subtelomere (Cytocell Ltd, UK) was included as a marker of the normal chromosome 1 and the der(3) chromosome.

Long-range PCR, Southern blot analysis, and Northern blot analysis

Long-range PCR was used for the amplification of the breakpoints and the generation of DNA probes for Southern blot analysis with an Advantage Genomic PCR kit (Clontech, USA). PCR was carried out following the manufacturer's user manual. Southern blot and Northern blot analyses were performed following the standard protocol. Human multiple tissue Northern blots

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were purchased from Clontech (Cat. #7780-1). Details of these analyses can be found in the Supplemental Experimental Procedures.

Mutation analysis

Mutation analysis of *LSAMP, NORE1A,* and *NORE1B* was performed in the 53 sporadic
5   CC-RCCs and 9 RCC cell lines. Each exon of *LSAMP, NORE1A,* and *NORE1B* was amplified by PCR using primers derived from the flanking intronic or UTR sequences (see Table A in the Supplemental Data). The PCR products were then purified and subjected to direct DNA sequencing using PE Applied Biosystems.

Real-time quantitative RT-PCR

10 Total RNA from normal kidney tissues, RCC cell lines, and sporadic tumors was subjected to real-time quantitative PCR using an ABI PRISM 7700 Sequence Detection System. Specific primer and probe were designed for *LSAMP* and *NORE1A* using Primer Express v1.5a (Applied Biosystems). The primer sequences and the details of the real-time RT-PCR analysis are described in the Supplemental Experimental Procedures.

15 **DNA methylation analysis and demethylation treatment by 5-aza-2'-deoxycytidine (5-aza-CdR)**

Methylation analysis was performed for the promoter CpG islands of *LSAMP* and *NORE1A*. Bisulfite-PCR followed by restriction enzyme digestion analysis was used. Eight RCC cell lines were demethylated by 5-aza-CdR (Sigma, USA) treatment. The primers and the details
20 of the analyses are given in the Supplemental Experimental Procedures.

LOH analysis

LOH detection for *LSAMP* and *NORE1* was performed by genotyping the 53 paired normal/tumor DNA samples. The microsatellite markers flanking the *LSAMP* locus are *D3S3681, D3S1271, D3S1267,* and *D3S1292. NORE1* locus markers include *D1S413* and
25 *D1S249.* All the markers were obtained from ABI Prism Linkage Mapping Set version 2, panel 1 and 2 (Applied Biosystems). The details of LOH analysis are described in the supplemental experimental procedures.

Cell growth assay

Expression plasmids *pEGFP-LSAMP, -NORE1A,* and *-Nore1* were generated by ligating
30 cDNAs of *LSAMP, NORE1A,* and murine *Nore1* to N- or C-terminal enhanced green fluorescent protein vectors (*pEGFP-N1/-C1*) (Clontech, USA). Expression plasmids were microinjected and transfected into two RCC cell lines, A-498 and/or Caki-1, for cell growth-suppression assay.

Inducible experiments and nuclear fractionation assays were also performed for the nuclear location of Nore1. Detailed methods are provided in the Supplemental Data.

URLs. University of California, Santa Cruz (UCSC) Human Genome Browser at the web site: www.genome.ucsc.edu; Discovery System Human Genome Browser: at the web site: www.cds.celera.com; Human Genome Browser NCBI: at the web site ncbi.nlm.nih.gov.

Supplemental Experimental Procedures

BAC clones used for construction of BAC contigs and FISH analyses

The 20 BAC clones from 1q32.1 are RP11-196B7, RP11-70G20, RP11-219P13, RP11-45F21, RP11-104A2, RP11-124A11, RP11-149C8, RP11-237N7, RP11-142B3, RP11-421E17, RP11-54L22, RP11-262N9, RP11-237C22, RP11-145I13, RP11-57I17, RP11-534L20, CTD-2245C1, CTD-2321B11, CTD-2278G17, and RP11-343H5.

The 24 BAC clones from 3q13.3 are RP11-138N21, RP11-58D2, RP11-324H4, RP11-165B13, RP4-635B5, RP11-484M3, RP11-829I14, RP11-641I23, RP11-643A3, RP11-891J4, RP11-281N16, RP11-50N14, RP11-728O20, RP11-899P8, RP11-716E6, RP11-1115L2, RP11-60P15, RP11-47C16, CTD-2246M24, RP11-149B11, CTD-2514L8, CTD-2016D14, CTC-804P8, and CTC-2006J5.

Long-range PCR, Southern blot analysis, and Northern blot analysis

Long-range PCR was used for the amplification of the breakpoints and the generation of DNA probes for Southern blot analysis with an Advantage Genomic PCR kit (K1906-Y, CLONTECH Laboratories, Inc., USA). PCR was carried out following the manufacturer's user manual. BAC clones spanning the 1q32.1 (RP11-54L22) and 3q13.3 (RP11-281N16) breakpoints were used as PCR templates. Four approximately 10-kb and five 4- to 6-kb DNA probes were synthesized for 1q breakpoint mapping and six 5- to7- kb DNA probes were generated for Southern blot analysis in 3q breakpoint mapping. Two EBV-transformed lymphoblastoid cell lines of two patients from the t(1q;3q) family and two normal EBV-transformed lymphoblastoid cell lines were used for Southern blot analysis. Fifteen microgram aliquots of genomic DNA were digested using *Bam*HI, *Eco*RI, *Hind*III, *Stu*I, *Eco*RV, *Xba*I, *Bgl*I, and *Bgl*II. Completely digested DNA samples were separated by size on a 0.8% agarose in 1 × TBE buffer. Southern blot to nylon membrane and subsequent hybridization were performed following the standard protocol.

For Northern blot analysis, human multiple tissue Northern blots were purchased from Clontech (USA, Cat. #7780-1). Northern blots were also prepared with RNA from normal

kidney (Clontech) and with RNA extracted from the nine RCC cell lines, the EBV lines FRCC3 and FRCC5, two EBV lines from normal individuals, and normal kidney tissues from two patients with sporadic CC-RCC. Total RNA was extracted using the Trizol Reagent kit (Invitrogen), and 15 µg of total RNA of each sample was for the Northern blots. Probes specific for *NORE1A* (exon 1α), *NORE1B* (exon 2β), *LSAMP* (exon 1), and β-actin were synthesized by PCR labeled with α-^{32}P and hybridized to the Northern filters under stringent conditions.

RT-PCR

RT-PCR was performed using 5 µg of total RNA isolated from the nine RCC cell lines and nine normal kidney tissues, Superscript-II RT (Invitrogen), random hexamer primers, and specific primer pairs. Specific primers from *LSAMP* and *NORE1A* were used for fusion transcripts analysis. The primer sequences are given in Table A of supplemental data online. PCR was carried out at 95°C for 5 min, followed by 95°C for 30 s, 58°C for 30 s and 72°C for 45 s, for 35 cycles.

LOH analysis

PCR was performed according to the manufacturer's protocol. For each individual, 1 µl of PCR product from each marker was then pooled. One microliter of this mixture was added to 10 µL of Hi-Di formamide and 0.1 µl of ROX 400HD size standard and denatured at 95 °C for 5 min before loading the samples into an ABI Prism 3700 DNA Analyzer (Applied Biosystems). Analysis of raw data and assessment of LOH were performed with Genescan v. 3.7 and Genotyper v. 3.7 software, respectively (Applied Biosystems). LOH was defined according to the following formula: LOH index = (T2/T1)/(N2/N1), where T was the tumour sample, N was the matched normal sample, and 1 and 2 were the intensities of smaller and larger alleles, respectively. If the ratio was less than 0.67 or more than 1.3, the result was determined to be LOH.

Real-time quantitative RT-PCR

Two micrograms of total RNA from 9 normal kidney tissues, the 9 RCC cell lines, and 16 sporadic tumors with *LSAMP* and/or *NORE1A* promoter methylation (RNA from other tumors was not available for analysis) were reverse-transcribed in a 100 µl reaction mixture using MultiScribe Reverse Transcriptase following the manufacturer's instruction (Applied Biosystems). Real-time quantitative PCR was performed using an ABI PRISM 7700 Sequence Detection System. Specific primer and probe sequences were designed for *LSAMP* and *NORE1A* using Primer Express v1.5a (Applied Biosystems).

LSAMP forward primer: 5'-CAATGGCCGTCCTGAACCT-3' (SEQ ID NO:106);

LSAMP reverse primer: 5'-CAAATTCCCTTCCAGTTGGTGTA-3' (SEQ ID NO:107);

LSAMP Taqman probe: 5'-6FAM-TTATCACCTGGAGACACC-MGBNFQ (SEQ ID NO:108);

NORE1A forward primer: 5'-GCGCTGCACTAACTGTAAATTCA-3' (SEQ ID NO:109);

5   *NORE1A* reverse primer: 5'-GGGATAAACCCTCCTGCTGACT-3' (SEQ ID NO:110);

NORE1A taqman probe: 5'-6FAM-TCACCCAGAATGCCGCA-MGBNFQ-3' (SEQ ID NO:111).

Of each sample, 100 ng was amplified using the following PCR conditions: 50°C for 2

min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. A series of

five 1:2 dilutions of pooled normal sample served as a standard curve for relative quantification.

10   Each tumor sample was normalized to an endogenous control, β-actin, and then normalized to

the standard curve. Reported are values of fold change from pooled normal.

DNA methylation analysis

Two µg of genomic DNA from each cell line, tumor and normal kidney tissue was

denatured in 0.3 M NaOH for 15 min at 37°C. Cytosines were sulfonated in 5 mM hydroquinone

15   (Sigma) and 3.12 M sodium bisulfite (Sigma) for 16 h at 50°C. The DNA samples were desalted

through columns, desulfonated in 0.3 M NaOH and precipitated with ethanol. DNA sequences

were amplified by nested PCR. Approximately 50 ng of bisulfite-treated DNA was firstly

amplified in a reaction volume of 30 µl with respective outer primer pairs:

LSAMP-BISF-OF (5'-TGGTAGAGGAGTATTTAGTTATAGAGAGA-3') (SEQ ID NO:112),

20   LSAMP-BISF-OR1 (5'-TCTCAATAAAACCAATAACAACTATTTC-3') (SEQ ID NO:113),

NORE1A-BISF-OF2 (5'-AAGAGGTAGGGTTGAAGGTTTAGGGTTT-3') (SEQ ID NO:114), and

NORE1A-BISF-OR2 (5'-CTCRAAACCRCTCAAACTCTATAAATAAC-3') (SEQ ID NO:115).

PCR was carried out at 95°C for 8 min, followed by 95°C for 30 s, 58°C for 30 s and 72°C for 1

min, for 30 cycles. A nested PCR was performed using 1 µl of the initially amplified products

25   and the respective internal primer pairs:

LSAMP-BISF-IF (5'-TGTTTGGGTTTTATGAGGGTTTTGT-3') (SEQ ID NO:116) and

LSAMP-BISF-IR (5'-CRACTAAACTCTCCTAACCATAATAACCAC-3') (SEQ ID NO:117),

NORE1A-BISF-IF2 (5'-GAATTTTGTAGTTGTTTTAGGTGAAGA-3') (SEQ ID NO:118), and

NORE1A-IR2 (5'-CRACRACTCRAAATCCAATAATAA-3') (SEQ ID NO:119)

30   with similar conditions as described for the preceding PCR amplification. The PCR products

were purified using Microcon YM-100 (Millipore Corporation, USA). For isoform *NORE1B*,

methylation analysis was performed as described in Tommasi *et al.* 2002. For restriction enzyme

analysis of PCR products from bisulfite-treated DNA, 30 ng of the PCR products was digested with 10 units of *Taq*I (Invitrogen, USA) and separated by size on a 2.0% TAE gel.

Demethylation of LSAMP and NORE1A by 5-aza-2'-deoxycytidine (5-aza-CdR) treatment

Eight RCC cell lines (except the slow-growing A-704 cell line) were subjected to 5-aza-CdR (Sigma Chemical Co., St. Louis, MO) treatment. Approximately 5×10^5 cells for each line were seeded on a 100-mm plate and incubated for 24 h. The cells were cultured up to 14 d in complete media which contained 2.5 μM of 5-aza-CdR, and media was changed at 2-d intervals. Isolated total cellular RNAs from RCC cell lines treated and untreated with 5-aza-CdR were analyzed with real-time quantitative RT-PCR.

Cell growth assay

RCC cell lines A-498 and/or Caki-1, growing logarithmically on glass coverslips and maintained in 10% fetal calf serum, were microinjected or lipid-mediated transfection with *pEGFP-LSAMP*, *pEGFP-NORE1A*, *pEGFP-Nore1* or the vector control *pEGFP-C1/pEGFP-N1* (50 ng/ml). Two hours after injection or 24 h after lipid-mediated transfection (Lipofectamine2000 reagents, Invitrogen), EGFP/EGFP-Nore1-expressing cells were fixed and stained with Texas Red-labeled phalloidin to reveal F-actin architecture and Hoechst 33342 (blue) to visualize DNA (nuclei). To monitor proliferation in cells expressing EGFP-LSAMP, EGFP-NORE1A, and Nore1 fusion proteins or EGFP, 40-60 cells were microinjected with expression plasmids for the indicated protein, and returned to the incubator for 2 h. The number of successfully injected/expressing cells were then counted on an inverted epifluorescence microscope and thereafter at the selected times.

Supplemental inducible experiments and nuclear fractionation assay for nuclear location of Nore1

To confirm the nuclear location of Nore1, me performed experiments by cloning Nore1 into the pIND(SP1)-Hygro vector (Invitrogen, Carlsbad, CA) and transfecting the plasmid into 293-T cells using Lipofectamine2000 reagents (Invitrogen). After selection in hygromycin, cell populations were pooled at an early passage and assayed for the effects of Nore1 induction on cell proliferation by growth curve analysis. Selected cells were plated at a cell density of 2.5 x 10^5 cells/well in triplicate and induced with Ponasterone A 24 h later. Cells were counted using a Coulter counter every 24 h. Western analysis was performed on lysates at each time point.

For nuclear fractionation assay, 293-T cells were transfected with 1 μg of pcDNAFlag-*Nore1* using CaPO4 (Invitrogen, Carlsbad CA). Forty-eight hours later cells were harvested and

processed for subcellular fractionation. Protein determinations were made for each fraction and equivalent amounts were loaded on to gel. The cytoplasmic, membrane and nuclear fractions were then subjected to Western analysis using an anti-FLAG antibody (Sigma, St. Louis. MO).

References for Examples

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From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make changes and modifications of the invention to adapt it to various usage and conditions.

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The preceding preferred specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever.

The entire disclosure of all applications, patents and publications, cited above and in the figures are hereby incorporated in their entirety by reference.